

**SHORT THESIS FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY (PHD)**

**Investigation of the *Penicillium chrysogenum* derived
antifungal protein (PAF) in animal models**

**by
Dr. Zoltán Palicz**

Supervisor:
Dr. Péter Szentesi



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By Dr. Zoltán Palicz MD

Supervisor: Dr. Péter Szentesi, PhD

Doctoral School of Molecular Medicine, University of Debrecen

Head of the **Examination Committee:** Prof. Dr. Zoltán Papp, MD, PhD, DSc

Members of the Examination Committee:

Prof. Dr. György Vereb, MD, PhD, DSc

Tamás Ivanics, MD, PhD

The Examination took place at Department of Physiology, Faculty of Medicine, University of Debrecen at May 22, 2017, at 11 AM.

Head of the **Defense Committee:** Prof. Dr. Zoltán Papp, MD, PhD, DSc

Reviewers: Dr. László Galgóczi, PhD

Dr. Zsuzsa Szigeti, PhD

Members of the Defense Committee: Prof. Dr. Miklós Geiszt, PhD, DSc

Dr. Judit Szabó, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at October 2, 2017, at 1 PM.

Introduction

Pulmonary aspergillosis is one of the most severe human diseases caused by fungi threatening especially immunodeficient patients. This expanding population is composed of patients with advanced HIV infection, prolonged neutropenia, and inherited immunodeficiency and patients who have undergone lung transplantation and allogeneic hematopoietic stem cell transplantation. The most severe form of pulmonary aspergillosis is invasive pulmonary aspergillosis, which leads to high mortality. In this disease the hyphae grow in the lung tissue, invading the blood vessels they can spread hematogenously to other organs causing fungal sepsis and death. For the treatment various antifungal drugs are used, from which the azoles are the most important (itraconazole, posaconazole, voriconazole). The mortality is also high in the treated patients, the cause of this can be the developing resistance of the fungi against the antifungal agents, which is increasing according to studies. In the case of azole resistance the fungi will be often resistant of more azoles simultaneously. Since the azoles are the most important drugs for treating aspergillosis, the spread of resistance against them is a serious problem during the treatment of immunocompromised patients. Therefore is an urgent task developing and introducing new antifungal drugs to human therapy. The antifungal protein produced by *Penicillium chrysogenum* (PAF) is a promising drug candidate. This protein was previously shown to be fungicide *in vitro* for some pathogenic fungi (*Aspergillus* and diverse dermatophytes), without toxic effects on mammalian cells.

Aims

We started to research the antifungal protein produced by *Penicillium chrysogenum* (PAF) due to the spread of the azole resistance among the *Aspergillus* species, to create a new antifungal drug which is effective against *Aspergillus* species. Since the protein had been examined *in vitro* in the aspect of toxicology and mycology, our aim was to explore the *in vivo* effects of PAF:

1. Examination of the potential toxic and proinflammatory effects of PAF in animal model by local and systemic administration.
2. Verification of the presence and function of PAF in the tissues of the treated animals.
3. Creating a stable animal model of invasive pulmonary aspergillosis.
4. Demonstration of the effectiveness of PAF in invasive pulmonary aspergillosis animal model with intranasal and intraperitoneal administration.
5. Verification of the advantages of coadministration of PAF and amphotericin B (AMB) in invasive pulmonary aspergillosis animal model.

Materials and methods

Purification of PAF

Purification of PAF was carried out as described previously. Briefly, *P. chrysogenum* NCAIM 00237 was grown in a sucrose (20 g·l⁻¹)–NaNO₃ (3 g·l⁻¹) minimal medium for 72 h at 25 °C with shaking. Mycelia were removed with centrifugation and the low molecular weight protein fraction of the supernatant was separated in Amicon Stirred Cells (Millipore, Billerica, MA, USA). PAF was purified with ion exchange chromatography on a CM Sephadex Fast Flow column (Amersham-Pharmacia, Uppsala, Sweden). The purity of the protein was tested on SDS-PAGE and by Western blotting. PAF was dissolved in physiological saline for the *in vivo* experiments.

Animal care

Animal experiments conformed with the guidelines of the European Community (86/609/EEC). The experimental protocol was approved by the institutional Animal Care Committee of the University of Debrecen. The mice were housed in plastic cages with mesh covers, and fed with pelleted mouse chow and water *ad libitum*. Room illumination was an automated cycle of 12 h light and 12 h dark, and room temperature was maintained within the range of 22–25 °C. Mice infected with *A. fumigatus* were kept in isolation cages with high-efficiency particulate air filters. These cages were continuously ventilated, and slightly negative air pressure was used to prevent infection from fungal spores. Each cage was inspected at least three times daily.

Administration of PAF

12 week old C57/Bl6 mice of both sexes were used in all *in vivo* experiments. First the mice were randomized into six groups (a control group and mice treated with 5, 25, 125, 250 and 2700 µg·kg⁻¹ PAF dissolved in sterile physiological (0.9%) saline;

10 animals per group) for the long time experiment and into two groups (control group and mice treated with 2700 µg·kg⁻¹ PAF; 5 animals per group) for the short time experiment. In the latter case the animals were treated daily for 2 weeks. To investigate the long term effect of PAF mice were treated once a week for 8 weeks. In this case we wanted to know if PAF provokes an immune response when applied for a longer period. Since PAF inhibited the growth of *A. fumigatus* by 95% in 125 µg·kg⁻¹ concentration, we applied it in a number of higher concentrations. Mice were slightly anesthetized with pentobarbital (50 mg·kg⁻¹)

intraperitoneally then the above solutions were administrated by pipettes into the nose of the animals in a 50 μL final volume. Under these conditions the solution reaches the lungs through normal breathing. The efficiency of the procedure was tested by applying a dye solution. The animals in the control groups were treated with 50 μL drug free physiological saline always. The weight of the mice was measured on the first and the last days of the short term application and once a week in the long term application.

Histological examination and lung injury score

To explore the possible side effects of short and long term application of PAF, the lungs, the kidneys, and the liver of the animals were examined histologically from control group and group treated with the highest (2700 $\text{g}\cdot\text{kg}^{-1}$) dose of PAF. The animals were killed on the last day of PAF treatment (14th or 56th day). The weight of the removed organs was measured. Then tissue samples were fixed in 8% buffered formalin (24 h) and embedded in paraffin wax (Shandon Pathcenter, Thermo-Shandon, USA). 4 μm sections were cut and Hematoxylin-eosin (H&E) staining was performed. The samples were investigated with a LEICA DM 2500 microscope equipped with a LEICA DFC 420 camera (Leica Microsystems, Germany) and the images were acquired using LEICA Application Suite (LAS) V3 software. The alveolar exudates/edema and hemorrhage were scored on a scale of 0–3 (0, absent; 1, mild; 2, moderate; 3, severe) from five randomly chosen fields in each sample.

Blood collection and cell counting

Blood samples were taken at the beginning and at the end of the two week long experiment from control group ($n = 16$) and group treated with the highest (2700 $\mu\text{g}\cdot\text{kg}^{-1}$) dose of PAF ($n = 10$). Mice were slightly anesthetized with pentobarbital and 200 μL blood was collected into a cup containing 40 μL acid citrate dextrose (ACD, Vacutainer tube, Becton Dickinson Diagnostics-Preanalytical Systems Plymouth, UK) by puncture of the retrobulbar venous plexus with a 1.5 mm diameter glass capillary. ACD anticoagulated whole blood was analyzed by Siemens Advia-120 hematology analyzer with Multi Species software (Deerfield, IL, USA), counting the number of the white blood cells and the neutrophil granulocytes. In addition, the level of liver enzymes and the ions in the plasma were measured to explore the possible toxic effects of PAF by Roche Integra 800 (Roche Diagnostics Mannheim, Germany).

Sample preparation for mass spectrometry and determination of relative PAF amounts in lung samples

PAF treated ($2700\ \mu\text{g}\cdot\text{kg}^{-1}$, $n = 5$) and control ($n=4$) animals were sacrificed and lungs were removed 4 h after the nasal administration of the solutions. The lungs were immediately frozen in liquid nitrogen and kept on $-70\ ^\circ\text{C}$ until analysis. The frozen lung tissue was homogenized and the proteins from the homogenate were acetone precipitated and re-dissolved in 25 mM ammonium bicarbonate. For protein concentration measurement the Bradford method was utilized. For trypsin digestion 100 μl sample and 0.25 ng trypsin was used at $37\ ^\circ\text{C}$ overnight and 10 μg protein was used for each mass spectrometry analysis.

Multiple reaction monitoring (MRM/SRM) based mass spectrometry method was developed for PAF measurement. The 1041.4/1038.6 and 1249.1/1245.9 PAF specific transitions were used. The MRM scans were performed on a 4000 QTRAP (ABSciex, Farmingham, USA) mass spectrometer using NanoSpray II MicroIon Source and controlled by the Analyst 1.4.2 software (ABSciex). The liquid chromatography was done on an EasynLC II nano HPLC (Bruker, Bremen, Germany) and the peptide mixture was separated on Zorbax 300SB-C18 column (Agilent, Santa Clara, USA) using a 30 min acetonitrile/water gradient at a flow rate of $300\ \text{nl}\cdot\text{min}^{-1}$. Specificity of the MRM/SRM transitions was tested by analyzing mouse lung tissue samples and their combination with 250 fmol PAF. For the estimation of PAF concentration the area under the curve (AUC) of the acquired spectra was calculated. The AUC were normalized to the protein content of the given lungs in each case. Those MRM spectra were used for AUC calculations where the intensity of the signal exceeded 150 cps (count per second) in both MRM transitions.

In vitro test of antifungal activity of PAF in the lung

Antifungal activity of PAF was tested using isolated lung tissue ($n = 3$). Lungs were removed 6 h after administration of $2700\ \mu\text{g}\cdot\text{kg}^{-1}$ PAF, homogenized with quartz sand in 900 μl physiological saline and centrifuged at $4\ ^\circ\text{C}$, 16,100 g for 10 min. The antifungal activity of the supernatant was determined on *A. nidulans* with micro-titer plate assay as described previously. Briefly, 10^3 conidia of *A. nidulans* spore suspension in 100 μl YPD liquid (1% yeast extract, 2% peptone, 2% glucose, pH 7.0) was incubated with 50–75–100 μl lung tissue homogenization overnight at $37\ ^\circ\text{C}$ in a micro-titer plate. The growth of *A. nidulans* was determined according to optical density at 630 nm. The antifungal activity of lung tissue from mice treated with PAF free physiological saline ($n = 3$) was also determined as control.

Model of LPS-induced acute lung inflammation

For induction of acute lung inflammation, C57/Bl6 mice were divided into 3 groups, slightly anesthetized with pentobarbital and physiological saline ($n = 4$), PAF ($2700 \mu\text{g}\cdot\text{kg}^{-1}$; $n = 4$), or lipopolysaccharide (LPS, *Escherichia coli* serotype 055:B5; Sigma-Aldrich; $250 \mu\text{g}\cdot\text{kg}^{-1}$; $n = 6$) was instilled intratracheally in a total volume of $50 \mu\text{l}$ physiological saline. This dose was based on previous studies. The treatment was not lethal, the animals recovered quickly from the procedure.

Measurement of pulmonary edema

To determine the pulmonary edema in acute lung inflammation, C57/Bl6 mice were divided into 3 groups. After a slight anesthetization with pentobarbital, physiological saline ($n = 4$), PAF ($2700 \mu\text{g}\cdot\text{kg}^{-1}$; $n = 4$), or lipopolysaccharide (LPS, $250 \mu\text{g}\cdot\text{kg}^{-1}$; $n = 4$) was instilled intratracheally in a total volume of $50 \mu\text{l}$ physiological saline. 24 h after the treatment the lungs were removed and the wet weights were measured. Lung tissues were weighed again after 3 days drying at 60°C . The wet-to-dry ratio was calculated as follows: $\text{wet-to-dry ratio} = (\text{wet weight} - \text{dry weight}) / \text{dry weight}$.

Positron emission tomography (PET)

The glucose analog ^{18}F FDG (2-[^{18}F]fluoro-2-deoxy-D-glucose) was synthesized and labeled with the positron-decaying isotope ^{18}F according to a previously described method. Images were collected using the MiniPET-II system, a dedicated small animal PET scanner. FDG-PET experiments were carried out on mice randomized into three groups (control, $n = 4$; PAF, $n = 4$; LPS, $n = 6$) on the next day of the intranasal application of solution. The animals were fasted for a minimum of 6 h before undergoing PET. After an injection of 4–6 mBq of ^{18}F FDG in $150 \mu\text{l}$ of phosphate buffered saline (PBS) via the tail vein, a dynamic PET scan was acquired using the small animal PET camera. Mice were maintained under isoflurane anesthesia during the injection and scanning periods. They were kept warm while under anesthesia to maintain a body temperature of 35°C . Three-dimensional regions of interest (ROI) were manually drawn around the edge of the lung activity by visual inspection using BrainCad software. The standardized uptake value (SUV) was calculated by multiplying the mean concentration activity in the ROI with mouse weight and dividing by injected dose.

Irritant dermatitis

The irritant dermatitis was evoked as described previously with minor modifications. Namely, stock solutions of PAF and phorbol-12-myristate-13-acetate (PMA) were prepared in physiological saline and DMSO, respectively and then both compounds were further diluted in hydrophilic cream (Unguentum hydrophylicum non-ionicum, Pharmacopoeia Hungarica VIII). Animals, 12 female C57/BL6 mice, were randomized into three groups (control, PMA, and PAF) and the thickness of their ears was measured. Mice were treated by smearing 4×10 μ L of vehicle (physiological saline), PMA (500 μ g/ml), or PAF (100 μ g/ml) onto the inner and outer surface of both ears. After 24 h, thickness of the ears was measured *in vivo* again. Finally the mice were sacrificed and tissue samples were taken for histology.

In vitro study assay

Minimum inhibitory concentration (MIC) determination of AMB and PAF and the drug combination studies were performed according to CLSI M38-A2 guidelines. The final concentrations of the tested drugs ranged from 0.0625 to 16 μ g/mL for AMB, and from 25 to 500 μ g/mL for PAF. The MICs of the antifungal agents were determined after 48 h of incubation at 35 °C as the lowest drug concentration at which there was complete inhibition of growth. In a checkerboard assay, combinations of the two drugs were determined according to the MIC values of the drugs used alone. The interaction of AMB and PAF was assessed by calculating the fractional inhibitory concentration index (FICI) with the following formula: $FICI = ((MIC\ A\ in\ combination) / MIC\ A) + ((MIC\ B\ in\ combination) / MIC\ B)$. The interaction is synergic if the FICI is ≤ 0.5 ; indifferent if the FICI is > 0.5 and ≤ 4 ; and antagonistic if the FICI is > 4 .

Preparation of fungal conidia

Inocula of *A. fumigatus* AF293 (clinical isolate from human lung tissue) were prepared by culturing the test organisms on minimal nitrate medium. Freshly grown (6 days) conidia were harvested in PBS-0.01% Tween 80 and counted in a Bürker chamber.

Infection of mice with *A. fumigatus*

Mice were immunosuppressed with 250 mg/kg cyclophosphamide (Endoxan, Baxter, IL, USA) intraperitoneally (IP) three days before and one day after infection with *A. fumigatus* conidia. A total of 3.5×10^6 conidia dissolved in 50 μ L PBS was given to mice intranasally

under anesthesia with isoflurane. The number of conidia was chosen to produce 100% mortality. This was tested in preliminary experiments. Gentamicin was added to the water of the mice (5mg/kg). The survival of the animals was monitored three times daily after infection. All infected animals were humanely terminated if they presented severely reduced mobility (i.e., unable to reach their water or food) or substantial distress. After the death of animals, their organs were removed and histological examination was carried out.

Intranasal PAF treatment of animals suffering from invasive pulmonary aspergillosis

In five independent experiments, immunosuppressed mice were randomly divided into three groups from which two were *Aspergillus* conidia infected. Among the infected groups the first group was treated with 5.4 mg/kg PAF (26 mice), the second group with PAF-free PBS (positive control, 23 mice) intranasally in the final volume of 50 μ L. The intranasal application of PAF was described earlier. Briefly, mice were slightly anesthetized with isoflurane, then 2.7mg/kg PAF dissolved in PBS were administered by pipette into the nose of the animals in a 50 μ L final volume. Under these conditions the solution reaches the lungs through normal breathing. Treatments lasted until the death of animals from the day of the infection twice a day (morning and afternoon). The control group (negative control, six mice) was not infected but immunosuppressed and treated intranasally with PAF-free PBS twice a day. Some animals from the infected group were killed 3 days after the infection, and their organs were examined histologically.

Intraperitoneal PAF treatment of healthy animals

Mice were randomly divided into two groups. The first group (n=8) was treated IP with 5.4 mg/kg PAF dissolved in 100 μ L PBS twice a day and intranasally twice a day with 2.7 mg/kg PAF dissolved in 50 μ L PBS for 7 days. The second (control) group (n=8) was treated with PAF-free PBS in the same way. On the eighth day, some animals were killed and the organs were removed for histological examination. On the first and last days of the experiment, body weight was measured, blood was collected, and blood cell count and other plasma parameters were determined.

Combined treatment with PAF and amphotericin B of animals suffering from invasive pulmonary aspergillosis

In two independent experiments, immunosuppressed mice were randomly divided into five groups (all together 11 mice per group). Animals in four groups were *Aspergillus* conidia

infected, the non-infected group was the negative control. All antifungal treatment started on the day of the fungal infection and lasted till the death of the animals. The first infected group was treated with PAF and amphotericin B-desoxycholate (Fungizone, Bristol-Myers Squibb, Montreal, QC, Canada), the second group with PAF, the third group with AMB and the positive control group only with PBS. A total of 5.4 mg/kg PAF dissolved in PBS was injected IP twice a day in 100 μ L final volume, and 50 μ L were administered by pipette into the nostrils of the animals under anesthesia once a day. One hundred microliters AMB were injected IP in 5 mg/kg concentration three times as follows: 1, 2 and 4 days after the fungal infection. The animals in the negative control group were treated only with PBS in the same way as the PAF treated group. After the death of the infected animals, their organs were removed for histological examination.

Chemicals and statistical analysis

Chemicals, unless otherwise stated, were purchased from Sigma (St. Louis, USA) and were of analytical grade. Averages were expressed as the means \pm standard error (SE) of the mean. The differences between control and treated animals were assessed using one-way analysis of variance (ANOVA) and all pairwise multiple comparison procedures (Student–Newman–Keuls method). The F test was used to test significance, and a P value of <0.05 was considered statistically significant. Percentage survival was computed with the Kaplan–Meier method. The mortality rate as a function of time was fitted to a Gompertz mortality rate equation. The equation is as follows: $R_m = (R_0 - pl) \times \exp(\alpha \cdot t)$,

where R_0 is the initial mortality rate, pl is plateau at the end of experiment, α is the Gompertz parameter and t is the time. The significance of differences between survival curves was assessed as the statistically accepted differences in the fitted Gompertz parameter (α) and with the log-rank (Mantel–Cox) test. The results were evaluated with GraphPad Prism 6.0 software (La Jolla, CA, USA).

Results

Histological examination and laboratory values of intranasally PAF treated animals

PAF was tested *in vivo* in several doses ($5\text{--}2700\ \mu\text{g}\cdot\text{kg}^{-1}$) either for a prolonged (8 weeks) or for a short period (2 weeks) in a high concentration ($2700\ \mu\text{g}\cdot\text{kg}^{-1}$) on mice. In case of the latter PAF was applied daily while in case of the former it was administered once a week. Since the most probable application of PAF in human therapy would be to treat aspergillosis which would require the drug to be introduced via the airways, presumably using inhalators, we attempted to simulate this condition by introducing PAF to mice via the airways. The drug was thus introduced after anesthesia through the nose while the applicability of the method, that is whether the liquid thus applied actually reached the alveoli, was confirmed in independent experiments. During the course of the experiments none of the animals died due to the drug application. Furthermore, the weight and physical activity of the animals remained unchanged during the study period. To test for possible morphological alterations due to the application of PAF, histological examination of the lungs, kidney, and liver of the animals was carried out. We did not observe any signs of pathological alterations in the lungs, in the kidney, and in the liver following the short (2 weeks, daily) application of the protein. Similar observations were made after the long (8 weeks, once a week) treatment, too. Both the total white blood cell count (1.94 ± 0.15 and $1.83 \pm 0.18\ 10^9\ \text{L}^{-1}$; control vs. treated, respectively; $F_{(1,8)} = 0.2$, $p = 0.66$) and the percentage of neutrophils (2.08 ± 0.57 and $2.10 \pm 0.29\%$; control vs. treated, respectively; $F_{(1,8)} = 0.001$, $p = 0.98$) remained unchanged in the highest dose ($2700\ \mu\text{g}\cdot\text{kg}^{-1}$) of PAF used daily for 2 weeks. Furthermore, the concentration of liver enzymes and ions in the blood did not show a significant elevation/alteration after PAF treatment. There was no significant change in the weight of the animals during the application of PAF. The average weight of the animals in the short term application of PAF was $21.6 \pm 0.2\ \text{g}$ at the beginning and $21.7 \pm 0.3\ \text{g}$ at the end ($F_{(1,8)} = 0.01$, $p = 0.92$), and $20.7 \pm 0.8\ \text{g}$ and $20.4 \pm 0.2\ \text{g}$ in the control group ($F_{(1,8)} = 0.17$, $p = 0.70$). The PAF treatment did not alter the growth of the animals in the long term (8 weeks) experiment. These results clearly established that the application of PAF neither induced any gross changes in organmorphology nor initiated an overall immune response in the animals.

Presence and activity of PAF in the lungs

To prove the efficiency of our method – that is to clearly establish that not only the solvent but also PAF was indeed present in the lungs following intranasal application – mass spectrometry was used to determine the presence of PAF in the lungs of PAF treated mice. The amount of PAF was calculated as the area under the curves of the mass spectrometry graphs. The relative amount (normalized to total protein content) of PAF was measured in control (n = 4) and PAF treated (n = 5) lung samples. The results demonstrate that the antifungal protein is clearly present in the lungs of PAF treated animals and its amount ($3.2 \pm 0.7 \text{ AUC} \cdot \text{mg}^{-1} \text{ protein}$) is significantly different from zero ($F_{(1,18)} = 22.121$, $p = 2.1 \cdot 10^{-3}$) and even significantly greater than that in the standard ($1.4 \pm 0.7 \text{ AUC} \cdot \text{mg}^{-1} \text{ protein}$, $F_{(1,12)} = 6.8$, $p = 0.023$). Note also that the values obtained for PAF content from untreated mice were not significantly different from zero ($0.010 \pm 0.007 \text{ AUC} \cdot \text{mg}^{-1} \text{ protein}$; $F_{(1,14)} = 0.197$, $p = 0.665$). To prove that PAF retained its antifungal activity in the lungs, homogenates of control and PAF treated mice lung were used in *in vitro* tests against *A. nidulans*. 100 μl of the supernatant of such homogenates inhibited the growth of *A. nidulans* approximately as a 50 $\mu\text{g} \cdot \text{ml}^{-1}$ PAF solution did. Namely, it reduced significantly the growth of *A. nidulans* to $25.2 \pm 1.6\%$ ($F_{(1,4)} = 3350.5$, $p = 5.3 \cdot 10^{-7}$; with 100% being the growth in culturing medium). Decreasing the amount of supernatant used in 75 μl or 50 μl resulted in increasingly smaller $39.4 \pm 1.2\%$ ($F_{(1,4)} = 3524.1$, $p = 4.8 \cdot 10^{-7}$) and $55.3 \pm 1.5\%$ ($F_{(1,4)} = 1184.5$, $p = 4.3 \cdot 10^{-6}$) but still significantly decreased growth, respectively. Lung tissue from untreated mice did not show any growth inhibitory effect on *A. nidulans* ($93.1 \pm 0.6\%$).

Inflammatory response in the lungs following PAF application

To test whether PAF application would induce inflammation of the lungs a novel non-invasive method based on ^{18}F FDG positron emission tomography was developed. The method is sensitive to all cellular processes that metabolize glucose since 18-fluorodeoxyglucose is taken up by glucose transporters into the cells and cannot be metabolized after its phosphorylation to ^{18}F FDG-6-phosphate. Its accumulation is, therefore, is proportional to the glucose uptake and, consequently, to the metabolic activity of the cell. FDG-PET experiments were carried out on 14 mice randomized into 3 groups. The 3D image of the body was reconstructed from the acquired images and the lungs were identified as Regions of Interest (ROI). The intensity of ^{18}F FDG accumulation within the ROI was assessed as a measure of inflammation. This method enabled us to give an estimate of and to follow any occurring inflammatory reactions in individual mice.

Mice were treated on the previous day with physiological saline, saline containing PAF (2700 $\mu\text{g}\cdot\text{kg}^{-1}$) or saline containing LPS (250 $\mu\text{g}\cdot\text{kg}^{-1}$) as positive control. It is clearly visible from the images that while PAF itself did not increase the emission intensity in the lungs as compared with control, LPS caused a marked elevation in the emission, indicating the presence of inflammation. These observations were quantified by calculating the emissions in the ROI. Pooled data showed a significant decrease in the PAF treated group (0.50 ± 0.01 SUV, $n = 4$, $F_{(1,6)} = 33.43$, $p = 0.01$) as compared with the control (0.72 ± 0.04 SUV, $n = 4$) and a significant elevation in the LPS treated animals (0.99 ± 0.09 SUV, $n = 6$, $F_{(1,8)} = 5.55$, $p = 0.04$). To validate the PET results, we sacrificed some animals from each group and conducted histological examination. H&E staining of the sections confirmed the absence of any inflammation in the control and the PAF-treated, while the presence of extensive inflammation in the LPS-treated animals. In addition the lung injury score was significantly higher in the LPS treated group (3.7 ± 0.5 , $F_{(1,11)} = 19.5$, $p = 0.001$) than in the control group (1.0 ± 0.4). On the other hand the score of PAF treated group (1.3 ± 0.3 , $F_{(1,11)} = 0.39$, $p = 0.54$) was almost identical to that of the control animals. Furthermore, unlike for LPS, PAF treatment was not accompanied by the development of pulmonary edema. In the simple assay for lung inflammation and pulmonary edema formation, LPS showed significantly increased lung wet-to-dry weight (4.04 ± 0.10 , $F_{(1,6)} = 11.041$, $p = 0.016$) as compared with the control (3.61 ± 0.07), while PAF did not elevate this ratio significantly (3.81 ± 0.10 , $F_{(1,6)} = 2.75$, $p = 0.15$) when examined 24 h after the treatment.

Inflammatory response of the skin following PAF application

Through the human application of PAF against aspergillosis would most likely be through inhalation, its surface application might also be considered, since PAF has inhibitory effects on several dermatophytes. To test for the possibility that PAF could induce local, contact inflammation on the skin, a contact dermatitis model was used where the swelling of the ears was measured. PAF treatment did not cause the swelling of the ears in the dose used. On the other hand, PMA, used as a positive control, induced a clear edema, indicating inflammation. This latter observation underlined that the model was sensitive enough to detect any inflammation. In particular, the increase in the thickness of ears of mice in the PMA group (57.5 ± 29.2 μm ; $n = 4$) was significantly greater ($F_{(1,10)} = 5.42$, $p = 0.04$) due to edema formation as compared to their control counterparts. In contrast, the increase in the thickness of the ears of PAF treated animals was essentially identical to that in the control group ($22.5 \pm$

5.0 and $23.8 \pm 9.2 \mu\text{m}$; in the control and in the PAF treated group, respectively, $n = 4$ for both groups, $F_{(1,10)} = 0.06$, $p = 0.81$).

Effects of intraperitoneal application of PAF on healthy animals

As the nasal application of PAF was not as successful as expected, the drug was administered IP as well. Control non-fungal-infected mice were treated IP with 0 and 5.4 mg/kg doses of PAF twice a day for 1 week. Blood samples were collected at the beginning and end of the experiment. Although the total white blood cell count decreased slightly ($F_{(1,14)}=35.5$, $P=3 \times 10^{-5}$), the percentage of neutrophils remained unchanged ($F_{(1,14)}=0.71$, $P=0.41$). Furthermore, the concentration of liver enzymes and ions in the blood did not show significant ($0.01 < F_{(1,14)} < 4.39$, $0.08 < P < 0.93$) elevation/alteration after PAF treatment. There was no significant change in the weight of the animals during the application of PAF. The average weight of the animals was 17 ± 0.3 g at the beginning and 17.2 ± 0.4 g at the end ($F_{(1,14)}=0.19$, $P=0.67$). These results suggest that the intraperitoneal application of PAF neither induced any overall changes in the animals nor initiated an overall immune response, similarly to the nasal application.

Survival and histological examination of animals suffering from pulmonary aspergillosis treated intranasally with PAF

As the most likely application of PAF in human therapy is expected to be the treatment of lung aspergillosis, which implies the administration of the drug via the airways, we simulated this procedure by introducing PAF intranasally to mice at a high concentration (5.4mg/kg). Infected animals suffering from IPA were evidently sick, moved slowly and their fur was matted. Moreover, their weight decreased significantly. PAF delayed the death of animals by ~ 1 day, but all animals had died by day nine. Log-rank analysis showed no significant differences ($P=0.86$) between the survival curves. In contrast with this, all of the control non-infected mice, treated only with PAF in PBS, showed 100% survival and their body weight was stable. Overall, intranasal PAF treatment did not increase significantly ($F_{(1,47)}=0.23$, $P=0.63$) the average survival of the animals. Histological examination of the lungs displayed severe pulmonary lesions characterized by multifocal to coalescent infiltrations of macrophages and neutrophils associated with vascular phenomena (thrombi, necrosis and hemorrhages) and alveolar and bronchiolar epithelial cell necrosis. There were no remarkably positive effects in the lungs following intranasal application of the antifungal protein. In positive control mice, a high number of proliferating hyphae and coagulation necrosis were

observed in the blood vessels, septa, bronchioles and alveoli. In the lungs from mice treated intranasally with PAF, hyphae were also frequently observed in the bronchioles and the alveoli. However, invasion of hyphae into blood vessels was not observed. The lung injury score was significantly higher in both cases (6 ± 0 and 5.3 ± 1.2 in positive control and PAF-treated mice, respectively) than in uninfected negative control animals (0.3 ± 0.3 ; $F_{(2,6)}=18.5$, $P=0.003$). The PAF-treated animals had values slightly but not significantly lower than the untreated ones ($F_{(1,4)}=0.31$, $P=0.61$). It is important to mention, however, that we did not find fungal infection in the kidneys or in the liver.

Combined treatment of animals suffering from pulmonary aspergillosis

To increase the positive effects of PAF, its intranasal application was complemented with intraperitoneal injection. The combined application of PAF and AMB was also tested. Before the *in vivo* application, an *in vitro* drug combination assay of AMB plus PAF was performed. With AMB and PAF alone, the MIC values against *A. fumigatus* AF293 proved to be 1 and 300 $\mu\text{g/mL}$, respectively. FICI values indicated an *in vitro* synergic effect when AMB was combined with PAF. The FICI value calculated from the individual and combined MICs (0.25 $\mu\text{g/mL}$ of AMB and 50 $\mu\text{g/mL}$ of PAF) of the drugs is 0.42. Infected mice were treated with PAF, amphotericin B (5 mg/kg), and both antifungal drugs concomitantly. It is clearly visible that although neither PAF nor AMB alone increased survival, the combined application resulted in a remarkable extension of life expectancy. In this experiment, all infected animals died within 7 days independently of the treatment they received. In the untreated infected group, more than 80% of the animals died after 5 days. Importantly, on this day more than 50% of the PAF or AMB-treated animals were still alive. It is noteworthy that in the group receiving the combined treatment, only 30% of the animals died by the 5th day. After 6 days, all PAF-treated animals died, meanwhile 9% of the AMB-treated animals and 27% of the combined treatment animals were alive. Summing up, individual PAF or AMB treatment extended the survival of the animals by 1 day, and some animals in the combined treatment group lived 2 days longer than those in the untreated group. To test the differences between the survival curves, Equation (1) was fitted to the data points. Although the Gompertz mortality rate was high ($\alpha=1.596$) in the positive control group, it was significantly lower in the PAF ($\alpha=0.6882$, $P<0.001$) and AMB ($\alpha=0.6146$, $P<0.001$) treated groups. Furthermore, the combined treatment significantly slowed down the mortality ($\alpha=0.4831$, $P<0.001$) when compared with the untreated group. As all animals died in all groups, the plateaus of the curves were assumed to be zero. The log-rank test also proved that the survival curves of the

treated groups are significantly different from that of the untreated one ($P=0.04$). There was no significant difference in the average survival time between the single treatment groups ($F_{(1,20)}=0.20$, $P=0.66$). PAF treatment alone caused a close to significant increase ($F_{(1,20)}=4.02$, $P=0.06$) in the average survival of the animals. However, AMB alone ($F_{(1,20)}=4.88$, $P=0.04$) and the combined PAF and AMB treatment ($F_{(1,20)}=6.99$, $P=0.02$) increased significantly the lifetime of the animals. These observations were validated by histological examination. In the lungs from IP PAF-treated mice, a number of proliferating hyphae and coagulation necrosis were noted in bronchioles and alveoli. In the lungs from mice treated with PAF and AMB, hyphae were not observed. H&E staining of sections confirmed less extensive inflammation in the combined AMB and PAF-treated animals. In addition, PAS staining proved the manifestation of extensive fungal infection in the single antimycotic (either PAF or AMB) treated animals and the lack of infection when PAF and AMB were used in combination. Furthermore, the lung injury score was lower in the combined treatment group (4 ± 0.6 , $F_{(1,5)}=4.96$, $P=0.08$) than in the control group (6.3 ± 0.8). However, the score of the PAF-treated group (5 ± 0.0) was almost identical to that of AMB-treated animals (5.3 ± 0.6 , $F_{(1,4)}=0.25$, $P=0.64$). None of them was significantly lower than the control value ($F_{(2,7)}=1.11$, $P=0.38$.)

Combined treatment with a reduced concentration of AMB

To investigate the efficacy of PAF, the previous experiment was repeated with a reduced concentration (2.5mg/kg) of AMB. The combined application of PAF and AMB ($n=6$) was compared with the single AMB-treated group ($n=11$). AMB alone delayed but did not prevent the death of the animals. After 8 days, all AMB-treated animals died, whereas its combined application with PAF not only delayed further the development of aspergillosis but also resulted in the survival of an infected mouse (16.6%). In contrast, all infected and untreated animals ($n=12$) died within 7 days. The combined PAF and AMB treatment extended the survival of the animals by 1 day, and one animal recovered from aspergillosis. Because the animals started to die earlier (at 2nd day) in the positive control group, the analysis of survival curves resulted in a slow ($\alpha=0.2822$) Gompertz mortality rate. It was significantly higher in AMB ($\alpha=0.4292$, $P=0.002$) treated groups, but the death of the animals started only at the 3rd day. In both cases the final number of animals (plateau) was 0. However, the combined treatment delayed the start of animal death to the 4th day, increased the survival of animals (plateau is 14.1%) and resulted in a significantly different mortality rate ($\alpha=0.4689$, $P=0.007$) when compared with the untreated group. However, the log-rank test only showed significant

difference for the trend ($P=0.03$) and not between the survival curves themselves ($P=0.09$). There was no significant difference in the average survival time between the single treatment and positive control group ($F_{(1,21)}=1.79$, $P=0.20$). However, the combined PAF and AMB treatment ($F_{(1,16)}=5.48$, $P=0.03$) increased the lifetime of the animals significantly.

Discussion

Aspergillus species have emerged as important causes of morbidity and mortality in immunocompromised patients. Despite the best treatment available, survival rate at 12 weeks is around 60–70% among patients with invasive pulmonary aspergillosis. Similar trend can be observed with dermatophytes which could spread when a big group of individuals regularly use communal changing rooms and swimming pools. Since the efficacy of the antifungal therapy is not complete in any type of fungal infections, the development and introduction of new antimycotic agents into human therapy would be beneficial beyond doubt. The Penicillium-derived antifungal protein (PAF) has been shown to be very effective in inhibiting the growth of several zoo-pathogenic fungi *in vitro*. The effects of PAF include hyperpolarization of the membranes of the fungal cells, as well as an increase in reactive oxygen species that oxidize intracellular proteins, lipids, and nucleic acids, causing not only the disintegration of the cellular membranes but also that of the mitochondria. These effects were achieved at the concentration of $10\ \mu\text{g}\cdot\text{ml}^{-1}$ for the most sensitive species and were present at $50\ \mu\text{g}\cdot\text{ml}^{-1}$ for all affected molds. The concentrations used in these experiments were thus determined on the basis of these and our earlier experiments, where membrane hyperpolarization and obvious signs of apoptosis in *Aspergillus* hyphae or protoplasts were observed after treatment with PAF. In our study we, therefore, used PAF concentrations of 5–2700 $\mu\text{g}\cdot\text{kg}^{-1}$ which corresponds to 2–1100 $\mu\text{g}\cdot\text{ml}^{-1}$. We thus expected that the highest concentration used in our study should be beyond the concentration that is sufficient to reach appreciable antifungal effects. This was indeed confirmed by testing the effects of lung homogenates of PAF treated mice on the growth of *A. nidulans* *in vitro*. In fact, two independent methods were used to establish the presence and antifungal efficiency of PAF in the lungs of PAF treated mice. First, mass spectroscopy experiments revealed that measurable amounts of the protein were present in the lungs of treated mice 4 h after the inhalation. Second, as mentioned above, the homogenate of these lungs successfully inhibited – by approximately 50–70% depending on the amount used – the growth of the *A. nidulans*, while lung homogenates from untreated mice had no such effects. These observations clearly demonstrated that not only was the protein still present in the lungs several hours after its introduction, but it retained its functional activity. It is also important to stress that mice treated with PAF showed no sign of any change in their behavior. They maintained regular activity as assessed by their normal water and food intake as well as the amount of spontaneous exercise they did (distance and speed of running in a voluntary activity wheel).

Furthermore, they kept their body weight or even gained weight during the long term application of the protein. These are clearly in line with our earlier *in vitro* studies where we found no toxic effects of PAF on mammalian cells, on neurons, on endothelial cells, and on skeletal muscle fibers in particular. Taken together, our present observation with *in vivo* application of PAF clearly suggests that this antifungal drug – in the concentration range used – has no effect on the overall condition of the treated animals. Several organs, including the lungs, the liver, the kidneys, and mucosa of the nose taken from the animals that received the highest (2700 $\mu\text{g}\cdot\text{kg}^{-1}$) concentration of PAF, underwent histological examination. The fact that no morphological changes were observed in these organs clearly argues that PAF has no toxic effects, however for the overall toxicity study the nervous, musculoskeletal, cardiovascular, and lymphoreticular systems should be examined, too. Our observation, in case of the liver, the kidneys, and the skeletal muscles, is substantiated by the finding that liver enzymes, the concentration of the important ions, and creatine kinase levels, respectively, in the blood of PAF treated animals were essentially identical to those of control animals. It must be mentioned, on the other hand, that a small downward trend was observed in the plasma parameters when treated with PAF. Since for some parameters (creatinin, total bilirubin, and liver enzymes) is the increase, while for others (albumin, Ca, K, and Na) both deviations could be pathological, a systematic toxic effect of the protein was not observed. This again argues that PAF treatment does not induce any detectable toxic effect in mice. In earlier experiments the possible *in vitro* pro-inflammatory action of PAF was examined on human blood. In those studies blood samples were incubated with different concentrations of PAF and the levels of IL-6, IL-8 and TNF- α were measured. The rise in the production of cytokines was minimal as compared with the effect of LPS. Here we examined whether the *in vivo* application of PAF would alter total white blood cell count and the relative number of neutrophil granulocytes. Results clearly established that the presence of PAF neither induced any change in the number of white blood cells nor did it alter the relative amount of neutrophils. In addition there was no neutrophil infiltration in histological samples of all organs examined. Particularly for the lung, the injury score was similarly low in the control and PAF treated group showing that hemorrhage was absent in both group of animals. However since a foreign protein was administered to mice, the possibility of immune responses could not be excluded. These observations are in line with the data presented using the FDG-PET technique. Since ^{18}F FDG accumulation in tissues is proportional to cell metabolism, it is, therefore, a clear marker for tissue inflammation. It was also shown that PET could be useful for monitoring activation of macrophages as well as neutrophils in early

lung inflammation. One would thus expect a clear increase in ^{18}F FDG signal in tissues where inflammation occurred. Our MiniPET-II system has been shown to be sensitive enough to detect such signals. Nevertheless, the application of PAF did not result in an increase in ^{18}F FDG related positron emission clearly indicating the lack of any inflammation of the lungs. On the other hand, LPS used as a positive control, initiated large increases in the SUV values similarly to those observed in LPS treated rats previously. It should be noted that in a few animals the lack or presence of inflammation as deduced from FDG-PET measurements was also confirmed by histological examinations substantiating our findings with ^{18}F FDG. Interestingly though SUV values were reduced in PAF treated animals as compared with their control counter mates. At the moment we do not have any clear explanation for this observation, nevertheless, similar results were obtained with some antineoplastic drugs in cell cultures. Earlier studies have demonstrated that PAF has inhibiting effects on several dermatophytes, which are a group of morphologically and physiologically related molds affecting the keratinous tissue of vertebrates. This raises the possibility that PAF could have a potential therapeutic use in dermatophytosis. Such application would inevitably require that local application of PAF on the skin and should not evoke any local inflammatory response. Measuring the changes of ear thickness following the surface application of different drugs has been an accepted model for detecting any inflammation in the skin. Our results from these experiments prove that PAF does not induce any inflammation if applied locally on the skin. This, therefore, raises the possibility that the protein could also be a potential candidate for the treatment of topical and systemic dermatophytosis. Taken together these results proved a good tolerance – no toxicity, no inflammation inducing effect – of PAF in several organs of mice, providing a basis for experiments evaluating the efficacy of PAF as a promising antifungal therapy.

We compared the *in vivo* antifungal activity of PAF when administered either intranasally or IP alone or in combination with amphotericin B — as an *in vitro* interaction study confirmed that the effect of these drugs is synergistic against *A. fumigatus* — in an immunocompromised mouse model of invasive pulmonary aspergillosis initiated by *A. fumigatus*. The model was highly reproducible, and all untreated animals died within 9 days following the fungal infection. Although PAF is a protein, it is highly stable, and, hence, it is easy and safe to prepare it for administration independent of the mode of application. In the case of nasal application, a concentration high enough to have antifungal activity *in vitro* was determined in the lungs. However, independent of the mode of application, the protein was undetectable in serum. Notably, accumulation of PAF over time was not observed, which was

most likely the consequence of its short half-life within the body. It should be noted that blood samples were taken 120 min after the last intraperitoneal administration of the drug and that PAF is small enough to be filtered by the glomeruli and thus excreted by the kidneys. On the basis of this, to achieve detectable concentrations of PAF in the serum of mice, the protein should be administered in doses higher than those used in our experiments. There may be a threshold therapeutic concentration for the successful medication of IPA, which should be determined in future studies. The intraperitoneal administration of PAF was beginning to prevent the spread of fungi in the lungs around days 5 and 6 post-infection, but finally it was not able to overcome the fungal invasion. It is remarkable that PAF alone was almost as effective as the lower (2.5 mg/kg) concentration AMB, an antimycotic used frequently in the medication of IPA. Furthermore, the combined application of PAF and AMB was clearly superior to either single antimycotic (PAF or AMB) treatment when increased survival times were considered. The dose of AMB (5mg/kg) used in this model was not able to prevent the progression of IPA in mice. In human treatment, AMB is usually administered in intravenous (iv.) infusion for 1–2 weeks, and its typical dosage is 3 mg/kg·day. With higher dosage of AMB (10 mg/kg·day), the incidence of nephrotoxicity increases. However, in patients with IPA, no difference was observed in efficacy between the low and high dosage of AMB showing ~ 50% positive effect. A comparative study with posaconazole and AMB found that although the survival with posaconazole was 70–90%, it was only 0–50% with AMB. The efficiency of AMB and anidulafungin was also compared, and both antimycotics could prolong survival, but finally at least 90% of the animals died in every group. These findings are in good accordance with our observations. We have to note notwithstanding that the intraperitoneal administration of AMB is less clinically relevant, but its synergic effects with PAF could also be investigated in our animal model. It was shown previously that the distribution of AMB in the lungs is significantly different when administered IP versus intravenously. As IP administration resulted in a higher level of AMB in the lungs than the iv. route, in further animal studies the applied concentration should be different than was used here. It is also possible that the histological changes we observed were partly the consequence of the accumulation of AMB, although we used AMB at a low concentration and only three times. It is also promising that the combined application of PAF with lowered AMB (2.5 mg/kg) was as effective or even better than the combined treatment with the high concentration AMB. This finding raises the possibility of combined treatment with reduced AMB and thus decreased toxicity, which has been observed in mice suffering from IPA. In contrast, no toxicities or side effects of PAF have been reported thus far. Similar to other

pathogenic fungi, *Aspergillus* spp. can develop resistance against different antifungal agents. For example, itraconazole resistance was first reported in 1997, and more than 50% of the strains resistant to itraconazole were cross-resistant to other azoles, such as voriconazole and posaconazole, which are primary drugs in antifungal therapies. Resistance to antimycotics can emerge through different mechanisms, such as increased drug reflux, modification or over-expression of target enzymes, up-regulation of homeostatic stress-response pathways, exogenous cholesterol import or altered drug uptake. Owing to the emergence and spread of resistance towards frequently used antifungals, there is an urgent need for new types of antimycotics effective against human opportunistic *Aspergillus* spp. Our study demonstrated for the first time that PAF has potential *in vivo* against IPA even when administered at relatively low concentrations. Furthermore, the fact that AMB interacted synergistically with PAF *in vitro* indicated that such combinations may also have good efficacy in the treatment of neutropenic patients suffering from IPA. Future *in vivo* studies should aim at shedding light on further interactions of antimycotic drugs with PAF and determining the threshold concentrations at which IPA could be blocked or even reversed.

Summary

The antifungal protein of *Penicillium chrysogenum* (PAF) inhibits the growth of important pathogenic filamentous fungi, including members of the *Aspergillus* family and some dermatophytes. PAF was proven to have no toxic effects on mammalian cells *in vitro*. To prove that PAF could be safely used in therapy, experiments were carried out to investigate its *in vivo* effects. Adult mice were inoculated with PAF intranasally in different concentrations, up to 2700 µg/kg daily, for 2 weeks. Even at the highest concentration -a concentration highly toxic *in vitro* for all affected molds used- animals neither died due to the treatment nor were any side effects observed. Histological examinations did not find pathological reactions in the liver, in the kidney, and in the lungs. Mass spectrometry confirmed that a measurable amount of PAF was accumulated in the lungs after the treatment. Lung tissue extracts from PAF treated mice exerted significant antifungal activity. Small-animal positron emission tomography revealed that neither the application of physiological saline nor that of PAF induced any inflammation while the positive control lipopolysaccharide did. The effect of the drug on the skin was examined in an irritative dermatitis model where the change in the thickness of the ears following PAF application was found to be the same as in control. Since no toxic effects of PAF were found in intranasal application, the next step was to test its efficacy against invasive pulmonary aspergillosis (IPA), experiments were carried out in mice suffering from IPA. Adult mice were immunosuppressed and then infected with *Aspergillus fumigatus*. After stable infection, the animals were inoculated with PAF intranasally at a concentration of 2.7 mg/kg twice per day. At this concentration -which is highly toxic *in vitro* to *A. fumigatus*- the mortality of the animals was slightly delayed but finally all animals died. Histological examinations revealed massive fungal infections in the lungs of both PAF-treated and untreated animal groups. Because intranasally administered PAF was unable to overcome IPA, modified and combined therapies were introduced. The intraperitoneal application of PAF in animals with IPA prolonged the survival of the animals only 1 day. Similar results were obtained with amphotericin B (AMB), with PAF and AMB being equally effective. Combined therapy with AMB and PAF -which are synergistic *in vitro*- was found to be more effective than either AMB or PAF treatment alone. As no toxic effects of PAF in mammals have been described thus far, and, moreover, there are so far no *A. fumigatus* strains with reported inherent or acquired PAF resistance, it is worth carrying out further studies to introduce PAF as a potential antifungal drug in human therapy.



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List of publications related to the dissertation

1. **Palicz, Z.**, Gáll, T., Leiter, É., Kollár, S., Kovács, I., Miszti-Blasius, K., Pócsi, I., Csernoch, L., Szentesi, P.: Application of a small molecular weight antifungal protein of *Penicillium chrysogenum* (PAF) against pulmonary aspergillosis in mice.
Emerg. Microbes Infect. 5 (11), e114, 2016.
IF: 4.012 (2015)
2. **Palicz, Z.**, Jenes, Á., Gáll, T., Miszti-Blasius, K., Kollár, S., Kovács, I., Emri, M., Márián, T., Leiter, É., Pócsi, I., Csósz, É., Kalló, G., Hegedűs, C., Virág, L., Csernoch, L., Szentesi, P.: In vivo application of a small molecular weight antifungal protein of *Penicillium chrysogenum* (PAF).
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List of other publications

3. Füzi, M., **Palicz, Z.**, Vincze, J., Cseri, J., Szombathy, Z., Kovács, I., Oláh, A., Szentesi, P., Kertai, P., Paragh, G., Csernoch, L.: Fluvastatin-induced alterations of skeletal muscle function in hypercholesterolaemic rats.
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